PATENT COOPERATION TREATY

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Woodcock Washburn Kurtz Mackiewicz & Norris LLP

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: MARK DELUCA
WOODCOCK, WASHBURN, KURTZ, MACKIEWICZ & NORRIS, LLP
ONE LIBERTY PLACE - 46TH FLOOR
PHILADELPHIA PA 19103

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PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

MT. (-c)

Date of Mailing *(day/month/year)*	25 JUN 2001

Applicant's or agent's file reference	IMPORTANT NOTIFICATION
UPAP-0377	

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/US00/11310	27 APRIL 2000	30 APRIL 1999

Applicant
THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	PHILLIP GAMBEL / TERRY J. DEY PARALEGAL SPECIALIST TECHNOLOGY CENTER 1600
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/416 (July 1992) ★

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference UPAP-0377	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/11310	International filing date *(day/month/year)* 27 APRIL 2000	Priority date *(day/month/year)* 30 APRIL 1999
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of _____ sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
25 OCTOBER 2000	07 JUNE 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PHILLIP GAMBEL TERRY J. DEY PARALEGAL SPECIALIST TECHNOLOGY CENTER 1600 Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (July 1998) ★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/11310

I. Basis of the report

1. With regard to the **elements** of the international application: *

☐ the international application as originally filed

☒ the description:
pages 1-66, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

☒ the claims:
pages 67-75, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

☒ the drawings:
pages 1-6, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

☒ the sequence listing part of the description:
pages NONE, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

☒ the description, pages NONE

☒ the claims, Nos. NONE

☒ the drawings, sheets/~~fig~~ NONE

5. ☐ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	1-7, 25-27	YES
	Claims	23, 24, 28-34, 36	NO
Inventive Step (IS)	Claims	1-7, 25-27	YES
	Claims	23, 24, 28-34, 36	NO
Industrial Applicability (IA)	Claims	1-7, 23-34, 36	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 23, 24 and 28-34 and 36 lack novelty under PCT Article 33(2) as being anticipated by GERSTMAYER et al. The GERSTMAYER et al reference teaches fusion proteins of the extracellular domain of CD80, which comprises the C domain, with immunoglobulin. The reference teaches vectors encoding the fusion protein. The teaching of GERSTMAYER et al constitutes a non-CD80 protein because it does not contain the entire CD80 sequence and the construct comprises a fusion partner which does not contain CD80 sequence. The teaching anticipates the claims.

Claims 1-7 and 25-27 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest constructs deleting the constant region of CD80, fusion proteins combining CD80 fragments with CD86 fragments or using the constructs for immunosuppression.

Claims 1-7, 23-34 and 36 meet the criteria set out in PCT Article 33(4) for industrial applicability.

---------- NEW CITATIONS ----------
NONE

PATENT COOPERATION TREA

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference UPAP-0377	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/11310	International filing date *(day/month/year)* 27 APRIL 2000	Priority date *(day/month/year)* 30 APRIL 1999
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA		

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   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

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   II ☒ Priority

   III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
25 OCTOBER 2000	07 JUNE 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PHILLIP GAMBEL TERRY J. DEY PARALEGAL SPECIALIST TECHNOLOGY CENTER 1600 Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (July 1998) ★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/11310

I. Basis of the report

1. With regard to the **elements** of the international application:*

- [ ] the international application as originally filed
- [X] the description:
  - pages 1-66 , as originally filed
  - pages NONE , filed with the demand
  - pages NONE , filed with the letter of _____
- [X] the claims:
  - pages 67-75 , as originally filed
  - pages NONE , as amended (together with any statement) under Article 19
  - pages NONE , filed with the demand
  - pages NONE , filed with the letter of _____
- [X] the drawings:
  - pages 1-6 , as originally filed
  - pages NONE , filed with the demand
  - pages NONE , filed with the letter of _____
- [X] the sequence listing part of the description:
  - pages NONE , as originally filed
  - pages NONE , filed with the demand
  - pages NONE , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

- [ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).
- [ ] the language of publication of the international application (under Rule 48.3(b)).
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3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- [ ] contained in the international application in printed form.
- [ ] filed together with the international application in computer readable form.
- [ ] furnished subsequently to this Authority in written form.
- [ ] furnished subsequently to this Authority in computer readable form.
- [ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- [ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:
   - [X] the description, pages NONE
   - [X] the claims, Nos. NONE
   - [X] the drawings, sheets/~~fig~~ NONE

5. [ ] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

International application No.
PCT/US00/11310

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

V. Reas n d statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	1-7, 25-27	YES
	Claims	23, 24, 28-34, 36	NO
Inventive Step (IS)	Claims	1-7, 25-27	YES
	Claims	23, 24, 28-34, 36	NO
Industrial Applicability (IA)	Claims	1-7, 23-34, 36	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 23, 24 and 28-34 and 36 lack novelty under PCT Article 33(2) as being anticipated by GERSTMAYER et al. The GERSTMAYER et al reference teaches fusion proteins of the extracellular domain of CD80, which comprises the C domain, with immunoglobulin. The reference teaches vectors encoding the fusion protein. The teaching of GERSTMAYER et al constitutes a non-CD80 protein because it does not contain the entire CD80 sequence and the construct comprises a fusion partner which does not contain CD80 sequence. The teaching anticipates the claims.

Claims 1-7 and 25-27 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest constructs deleting the constant region of CD80, fusion proteins combining CD80 fragments with CD86 fragments or using the constructs for immunosuppression.

Claims 1-7, 23-34 and 36 meet the criteria set out in PCT Article 33(4) for industrial applicability.

---------- NEW CITATIONS ----------
NONE

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/11310

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/11310

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-22, drawn to recombinant C domain-deficient CD80 polypeptides and a method of immunizing an individual therewith.

Group II, claims 23-40, drawn to non-CD80 polypeptides comprising CD80 C domain and a method of immunosuppressing an individual therewith.

The inventions listed as Groups I & II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The recombinant constructs of Group I all share the salient feature that they lack the constant region of the CD80 protein, also known as B7.1, while the constructs of Group II each require that the constant region of CD80 is present. Further, the method of Group I, using the compound of that group, is drawn to the stimulation of an immune response in the individual, while the method utilizing the construct of Group II is drawn to inhibiting an immune response in a subject. Therefore, the groups are not linked by a special technical feature so as to constitute a single inventive concept.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/11310

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 45/00; A01N 37/18; C07K 1/00
US CL : 530/350, 403, 806, 868; 424/287.1; 514/2, 885
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350, 403, 806, 868; 424/287.1; 514/2, 885

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, DIALOG medicine index:
terms -- CD80, CD86, B7.1, B7.2, constant, recombinant, fusion, mutant, immuniz$, immunosuppress$, deletion

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US 5,942,607 A (FREEMAN et al) 24 August 1999, see entire document.	1-15, 23-37
X --- Y	LANIER et al. CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. J. Immunol. 01 January 1995, Vol. 154, No. 1, pages 97-105, especially abstract.	23-27 ---- 1-15, 28-37
Y	LINSLEY et al. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. Immunity. December 1994, Vol. 1, No. 9, pages 793-801, see entire document.	1-15, 23-27

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 08 JULY 2000	Date of mailing of the international search report 17 OCT 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer F. PIERRE VANDERVEGT Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/11310

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GERSTMAYER et al. Costimulation of T-cell proliferation by a chimeric B7-antibody fusion protein. Cancer Immunol. Immunother. 1997, Vol. 45, No. 3-4, pages 156-158, see entire document.	1-15, 23-37

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 08 February 2001 (08.02.01)	
International application No. PCT/US00/11310	**Applicant's or agent's file reference** UPAP-0377
International filing date (day/month/year) 27 April 2000 (27.04.00)	**Priority date** (day/month/year) 30 April 1999 (30.04.99)
Applicant WEINER, David, B. et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   25 October 2000 (25.10.00)

   [ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

   [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des C lombettes** **1211 Geneva 20, Switzerland**	Authorized officer A. Karkachi
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 November 2000 (09.11.2000)

PCT

(10) International Publication Number
WO 00/66162 A1

(51) International Patent Classification[7]: A61K 45/00, A01N 37/18, C07K 1/00

(21) International Application Number: PCT/US00/11310

(22) International Filing Date: 27 April 2000 (27.04.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/131,764 30 April 1999 (30.04.1999) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 60/131,764 (CIP)
Filed on 30 April 1999 (30.04.1999)

(71) Applicant *(for all designated States except US)*: **THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA** [US/US]; 3700 Market Street, Suite 300, Philadelphia, PA 19104 (US).

(71) Applicants and
(72) Inventors: **SEKALY, Rafick, P.** [CA/CA]; 2740 O'Grady, St. Laurent, Quebec, H4M 2S2 (CA). **HOLTERMAN, Mark** [US/US]; University of Illinois, 840 S. Wood Street, Chicago, IL 60612 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **WEINER, David, B.** [US/US]; 717 Beacom Lane, Merion Station, PA 19066 (US). **AGADJANYAN, Michael, G.** [RU/US]; 353 Kevin Court, Philadelphia, PA 19116 (US).

(74) Agent: **DELUCA, Mark**; Woodcock Washburn Kurtz Mackiewicz & Norris LLP, One Liberty Place, 46th Floor, Philadelphia, PA 19103 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(48) Date of publication of this corrected version:
10 January 2002

(15) Information about Correction:
see PCT Gazette No. 02/2002 of 10 January 2002, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/66162 A1

(54) Title: MUTANT HUMAN CD80 AND COMPOSITIONS FOR AND METHODS OF MAKING AND USING THE SAME

(57) Abstract: Improved vaccines and methods of using the same are disclosed Immunosuppressive compositions for treating individuals who have autoimmune diseases or transplants and methods of using the same are disclosed.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
A61K 45/00, A01N 37/18, C07K 1/00

A1

(11) International Publication Number: WO 00/66162

(43) International Publication Date: 9 November 2000 (09.11.00)

(21) International Application Number: PCT/US00/11310

(22) International Filing Date: 27 April 2000 (27.04.00)

(30) Priority Data:
60/131,764 30 April 1999 (30.04.99) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 60/131,764 (CIP)
Filed on 30 April 1999 (30.04.99)

(71) Applicant *(for all designated States except US)*: THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; 3700 Market Street, Suite 300, Philadelphia, PA 19104 (US).

(71)(72) Applicants and Inventors: SEKALY, Rafick, P. [CA/CA]; 2740 O'Grady, St. Laurent, Quebec, H4M 2S2 (CA). HOLTERMAN, Mark [US/US]; University of Illinois, 840 S. Wood Street, Chicago, IL 60612 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: WEINER, David, B. [US/US]; 717 Beacom Lane, Merion Station, PA 19066 (US). AGADJANYAN, Michael, G. [RU/US]; 353 Kevin Court, Philadelphia, PA 19116 (US).

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(54) Title: MUTANT HUMAN CD80 AND COMPOSITIONS FOR AND METHODS OF MAKING AND USING THE SAME

(57) Abstract

Improved vaccines and methods of using the same are disclosed Immunosuppressive compositions for treating individuals who have autoimmune diseases or transplants and methods of using the same are disclosed.

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MUTANT HUMAN CD80 AND COMPOSITIONS FOR AND METHODS OF MAKING AND USING THE SAME

FIELD OF THE INVENTION

The present invention relates to compositions for and methods of immunizing individuals, to immunosuppressive compositions, components thereof and methods of making and using the same.

BACKGROUND OF THE INVENTION

This application is related to U.S. Provisional Application Serial Number 60/131,764 filed April 30, 1999, which is incorporated herein by reference.

CD28 is a cell surface glycoprotein constitutively expressed on most mature T-cells and thymocytes, while the CTLA-4 receptor is not present on resting T cells and is only detectable 48 to 72 hours after T cell activation. The principal ligands for CD28/CTLA-4 molecules are B7.1 (CD80) and B7.2 (CD86) expressed on the surface of professional antigen presenting cells (APC). The biological rationale for the existence of at least two receptors (CD28 and CTLA-4) and two ligands (CD80 and CD86) is not clear. It was initially demonstrated that CD80 and CD86 antigens were functionally similar. However, different roles for these co-stimulatory molecules were first suggested when the different patterns of their expression were determined. CD86 is constitutively expressed on APC and after activation of APC, the expression of CD86 is quickly up-regulated followed by a gradual return to baseline levels. The expression of CD80 is delayed compared to

CD86 and its expression is maximal 48 to 72 hours after the initiation of an immune response. Because CD86 expressed constitutively and up-regulated earlier than CD80 it was suggested that CD86 expression is important for the early phase of an immune response, while CD80 is important for the second.

Functional differences between CD80 and CD86 are further suggested by data on the binding kinetics of co-stimulatory molecules with CD28 and CTLA-4. Surface plasmon resonance (SPR) analysis has demonstrated that both ligands bind to CTLA-4 with higher avidity than to CD28. Further measurements revealed that the CD86/CTLA-4 complex dissociates faster than the CD80/CTLA-4 complex. These binding differences combined with the similar delay in expression of CTLA-4 and CD80 suggest that functional relationship between CTLA-4 and CD80 is probably more potent than functional relationship between CTLA-4 and CD86 molecules.

Multiple functions for CD80 and CD86 molecules *in vitro* and *in vivo* have been also reported. Anti-CD86 but not anti-CD80 antibodies block the development of disease in a mouse model of autoimmune diabetes, whereas the opposite effect is seen with these antibodies in a murine model of experimental allergic encephalomyelitis. Several experimental systems demonstrate an important role for CD86 in initiating a T-cell response to antigen and that the CD80 molecule may play an important role in providing modulatory signals to these cells. It was observed that expression of exogenous human CD86, but not CD80, provides important activation signals to murine T cells following DNA vaccination with envelope proteins from HIV-1. Similar results were observed after immunization of mice with DNA encoding HIV-1 or influenza antigens and plasmids encoding murine CD80 and CD86. Thus, functional differences between CD80 and CD86 were not connected with differential immunogenicity of human costimulatory molecules expressed in the mouse organism. It is believed that exogenous human or murine CD86, but not CD80, stimulates anti-viral T-cell activation during DNA immunization.

Vaccines are useful to immunize individuals against target antigens such as pathogen antigens or antigens associated with cells involved in human diseases. Antigens associated with cells involved in human diseases include cancer-associated tumor antigens and antigens associated with cells involved in autoimmune diseases.

In designing such vaccines, it has been recognized that vaccines which produce the target antigen in the cell of the vaccinated individual are effective in inducing the cellular arm of the immune system. Specifically, live attenuated vaccines, recombinant vaccines which use avirulent vectors and DNA vaccines all lead to the production of antigens in the cell of the vaccinated individual which results induction of the cellular arm of the immune system. On the other hand, sub-unit vaccines, which comprise only proteins, and killed or inactivated vaccines induce humoral responses but do not induce good cellular immune responses.

A cellular immune response is often necessary to provide protection against pathogen infection and to provide effective immune-mediated therapy for treatment of pathogen infection, cancer or autoimmune diseases. Accordingly, vaccines which produce the target antigen in the cell of the vaccinated individual such as live attenuated vaccines, recombinant vaccines which use avirulent vectors and DNA vaccines are often preferred.

While such vaccines are often effective to immunize individuals prophylactically or therapeutically against pathogen infection or human diseases, there is a need for improved vaccines. There is a need for compositions and methods which produce an enhanced immune response.

Gene therapy, in contrast to immunization, uses nucleic acid molecules that encode non-immunogenic proteins whose expression confers a therapeutic benefit to an individual to whom the nucleic acid molecules are administered. A specific type of gene therapy relates to the delivery of genetic material which encodes non-immunogenic proteins that modulate immune responses in the individual and thus confer a therapeutic benefit. For example, protocols can be designed to deliver genetic material which encodes non-immunogenic proteins that downregulate immune responses associated with an autoimmune disease in an individual and thus confer a therapeutic benefit to the individual. There is a need for compositions and methods which can be used in gene therapy protocols to modulate immune responses.

Modulation of immune responses by alternative means is similarly desirable to treat diseases such as autoimmune disease and cell/tissue/organ rejection. There is a need for compositions and methods which can be used to modulate immune responses and to design and discover compositions useful to modulate immune responses.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows data from experiments described in the Example showing antigen specific anti-viral CTL responses.

Figures 2A and 2B show data from experiments described in the Example showing lymphokine production induced by various constructs.

Figure 3 shows data from experiments described in the Example showing CTL activity following administration of constructs.

Figure 4 shows data from experiments described in the Example showing CTL activity following administration of constructs measured after the removal of this population of cells.

Figure 5 are photographs from experiments described in the Example showing infiltration of lymphocytes into the muscle of mice immunized with constructs.

Figure 6 is a graphic representation of the CD80 molecule.

DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

Applicants have discovered that the C region of human CD80 is responsible for transmuting a negative signal when an antigen presenting cell (APC) interacts with a T cell. The negative signal results in a reduction in the activity of the T cell and thus a reduction in the immune response generated against the antigen presented by the APC to the T cell. Specifically, the interaction between a T cell receptor (TCR) on a T cell with an MHC/antigen complex that has been formed on an APC by the formation of a complex between a major histocompatibility complex (MHC) protein and an antigen is accompanied by the interaction between the co-stimulatory molecules CD80 and CD86 present on the APC with CD28 molecules on the T cell. Such interaction results in T cell activation and an elevated immune response. However, following T cell activation, CTLA4 is expressed by T cells. CTLA4 interacts with CD80 and such interaction results in a dominant negative signal which eliminates the previous co-stimulatory effect caused by CD80 and CD86 interaction with CD28. The result of CD80 interaction with CTLA4 is a reduction in the immune response with which the T cell is involved.

Applicants discovery provides for two distinct aspects of the invention. According to one aspect of the invention, CD80 mutants and nucleic acids encoding the

same are provided which possess the co-stimulatory activity of CD80 but which do not transmute the negative signal associated with CD80 interaction with CTLA4. Such CD80 mutants are useful in immunization protocols in which they are delivered, as proteins or nucleic acids encoding such proteins, together with immunogens which are delivered as protein immunogens or nucleic acid molecules encoding immunogens. The CD80 mutants of this aspect of the invention are molecular adjuvants in immunization protocols. According to another aspect of the invention, CD80 mutants and nucleic acids encoding the same are provided which possess the CD80 C region so that they transmute the negative signal associated with CD80 interaction with CTLA4. Such CD80 mutants are useful in the treatment of autoimmune diseases and immunosuppression protocols associated with cell, tissue and organ transplants. The CD80 mutants which provide the negative signal may be delivered as proteins or nucleic acids encoding such proteins. The CD80 mutants of this aspect of the invention are autoimmune/immunesuppressive therapeutics.

The nucleotide and amino acid sequences of human CD80 is well known and set forth in Freeman et al. (1989) J. Immunol. 143(8):2714-2722, Selvakumar et al. (1992) Immunogenetics 36(3):175-181, Freeman et al. (1991) J. Ex. Med. 174(3):625-631, Lanier et al. (1989) J. Immunol. 154(1):97-105, and Genbank accession code P33681 (www.ncbi.nlm.nih.gov), which are each incorporated herein by reference.

CD86 (B7.2) was first described in Azuma, M. et al. 1993 Nature 366:76-79, which is incorporated herein by reference. Figure 2B of that publication discloses the nucleotide and predicted amino acid sequence of the B7.2 protein. The sequence information is also available in the Genbank database as U04343 which is incorporated herein by reference.

Human CD80 is expressed as a 288 amino acid protein (1-288) which is processed to a mature protein (35-288). CD80 is divided into four regions: the variable (V) region, the constant (C) region, the transmembrane region (tm) and the cytoplasmic tail region (ct). Amino acids 35-242 make up the extracellular domain of the protein. Amino acids 43-123 make up the V region, also referred to as the Immunoglobulin like V-type domain. Amino acids 155-223 make up the C region, also referred to as the Immunoglobulin like C2-type domain. Amino acids 243-263 make up the transmembrane region. Amino acids 264-288 make up the cytoplasmic tail.

As used herein, the terms "CD80 mutants", "C region- CD80 mutants", "C region deficient CD80 mutants" and "CD80ΔC mutants"are used interchangeably and are meant to refer to molecules which contain either a functional CD80 or CD86 V region, at least one functional non-C region of CD80 and which are free of a functional C region of CD80 such that, through the absence of all or part of the C region, such molecules do not transmute the negative signal associated with wild type CD80 C region interactions with CTLA4.

As used herein, references to a "functional region of CD80" as used in the phrases to "at least one functional non-C region of CD80" and "functional C region of CD80" are meant to refer to complete protein regions from CD80 as well as partial regions which retain the activity of the complete region. For example, a functional V region of CD80 refers to amino acids 43-123 of CD80 or a fragment thereof, including proteins which include other sequences including but not limited to other CD80 sequences, which retains the ability to bind to CD28. A functional C region of CD80 refers to amino acids 155-223 of CD80 or a fragment thereof, including proteins which include other sequences including but not limited to other CD80 sequences, which retains the ability to bind to CDLA-4 and transmute a negative signal. Thus a protein free of a functional C region of CD80 may contain a fragment of amino acids 155-223 of CD80 but such a fragment does not bind to CDLA-4 and transmute a negative signal. Similar proteins free of a functional C region may include amino acids 155-223 if adjacent sequences are changed to render the C region non-functional through conformational or other changes. A functional tm of CD80 refers to amino acids 243-263 of CD80 or a fragment thereof which retains the anchor a mutant CD80 protein which comprises it into the cell membrane and thereby prevent secretion. A functional ct of CD80 refers to amino acids 264-288 of CD80 or any fragment thereof which is retained in the cytoplasm when the mutant CD80 protein is expressed.

As used herein, the terms "C region$^+$ CD80 proteins" and "C region proteins" are used interchangeably and are meant to refer to those proteins which comprise a functional CD80 C region and due to the presence of all or part of the C region, such molecules transmute the negative signal associated with wild type CD80 C region interactions with CTLA4.

One aspect of the present invention relates to improved methods and compositions for vaccination, particularly DNA vaccination in which DNA that encodes target immunogens is administered into the individual in whom the DNA is taken up and expressed and an immune response is generated against the immunogen. According to aspects of the invention, DNA that encodes a CD80ΔC mutant protein is co-delivered to the individual and the expression of such DNA produces the CD80ΔC mutant protein which enhances the immune response induced against the immunogen.

It has been discovered that the co-production of CD80ΔC mutant protein in cells of a vaccinated individual that are expressing target antigens results in an surprisingly enhanced immune response against the target antigen. By providing an expressible form of nucleotide sequence that encodes CD80ΔC mutant proteins, vaccines which function by expressing target antigen in the cells of the vaccinated individual, such as DNA vaccines, recombinant vector vaccines and attenuated vaccines, the vaccines are improved.

The co-production of CD80ΔC mutant proteins in cells producing antigens results in enhanced cellular immunity against the antigen. Accordingly, the present invention provides improved vaccines by providing a nucleotide sequence that encodes CD80ΔC mutant protein operably linked to necessary regulatory sequences for expression in vaccinees as part of vaccines such as DNA vaccines, subunit avirulent recombinant vector vaccines and live attenuated vaccines. Alternatively, CD80ΔC mutant protein is delivered as a protein adjuvant together with an immunogen or gene construct encoding an immunogen.

According to some embodiments of the invention in which CD80ΔC mutants are provided as molecular adjuvants in immunization protocols, the CD80ΔC mutants contain either a functional CD80 V region or a functional CD86 V region. The CD80ΔC mutants do not contain a functional CD80 C region. In some embodiments, the C region is deleted and the V region is linked directly to the transmembrane region. In some embodiments the CD86 C region is inserted in place of the CD80 Cregion. In some embodiments, non-CD-80, non-CD86 sequences are included in the CD80ΔC mutants after the V region. Some embodiments include the CD80 transmembrane region. Some embodiments include the CD86 transmembrane region. In some embodiments, the CD80 transmembrane region is deleted and not substituted by any other sequences. Some

embodiments include non-CD-80, non-CD86 sequences in place of the CD80 tm. Some embodiments include the CD80 cytoplasmic tail. Some embodiments include the CD86 cytoplasmic tail. In some embodiments, the CD80 cytoplasmic tail is deleted and not substituted by any other sequences. Some embodiments include non-CD-80, non-CD86 sequences in place of the CD80 ct. It has been discovered that in those embodiments in which the CD80ΔC mutants are delivered to the cells by the administration of genetic material which encodes the CD80ΔC mutant, those CD80ΔC mutants which include a transmembrane region and cytoplasmic tail are particularly effective in stimulating immune responses. In some embodiments, the CD80 tm and CD80 ct are provided. In some embodiments, the CD86 tm and CD86 ct are provided. In some embodiments, the CD80 tm and CD86 ct are provided. In some embodiments, the CD86 tm and CD80 ct are provided. In those embodiments in which the CD80ΔC mutants are delivered to the cells by the administration of CD80ΔC mutant proteins, the CD80ΔC mutant proteins may be provided as a soluble protein in which the transmembrane region and cytoplasmic tail are deleted and, in some cases, replaced with a soluble moiety.

Aspects of the present invention relate to isolated proteins that comprises80V, 80tm and 80ct and is free of 80C; wherein said protein comprises either 80V or 86 V or both and optionally comprises one or more of 80tm, 86tm, 80ct and 86ct and wherein:

80V is the variable domain of CD80 or a functional fragment thereof;
86V is the variable domain of CD86 or a functional fragment thereof;
86C is the C domain of CD86 or a functional fragment thereof;
80tm is the transmembrane region of CD80 or a functional fragment thereof;
86tm is the transmembrane region of CD86 or a functional fragment thereof;
80ct is the cytoplasmic tail of CD80 or a functional fragment thereof; and
86ct is the cytoplasmic tail of CD86 or a functional fragment thereof.

According to some embodiments, the have the formula:

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8\text{-}R^9$$

wherein

R^1 is 0-50 amino acids;

R^2 is 80V or 86V;

R^3 is 0-50 amino acids;

R^4 is 86C or 0 amino acids;

R^5 is 0-50 amino acids;

R^6 is 80tm or 86tm;

R^7 is 0-50 amino acids;

R^8 is 80ct or 86ct; and

R^9 is 0-50 amino acids.

In some embodiments R^1 is 0-25 amino acids; R^3 is 0-25 amino acids; R^5 is 0-25 amino acids; R^7 is 0-25 amino acids; and/or R^9 is 0-25 amino acids.

In some embodiments R^1 is 0-10 amino acids; R^3 is 0-10 amino acids; R^5 is 0-10 amino acids; R^7 is 0-10 amino acids; and/or R^9 is 0-10 amino acids.

In some embodiments, the protein is a CD80 mutant selected from the group consisting of:

80V/dele/80tm/80ct;

80V/dele/80tm/86ct;

80V/dele/86tm/80ct;

86V/dele/80tm/80ct;

86V/dele/80tm/86ct;

86V/dele/86tm/80ct;

80V/dele/86tm/86ct;

80V/86C/80tm/80ct;

80V/86C/80tm/86ct;

80V/86C/86tm/80ct;

86V/86C/80tm/80ct;

86V/86C/80tm/86ct;

86V/86C/86tm/80ct;

80V/86C/86tm/86ct;

80V/dele/80tm/dele;

80V/dele/86tm/dele;

86V/dele/80tm/dele;

80V/86C/80tm/dele;

80V/86C/86tm/dele;

86V/86C/80tm/dele;

86V/86C/80tm/dele;

86V/86C/dele/80ct;

80V/86C/dele/80ct;

80V/dele/dele/80ct;

86V/dele/dele/80ct;

80V/86C/dele/dele; and;

80V.

In some embodiments, the CD80 mutant has the formula selected from the group consisting of:

R-80V-R-dele-R-80tm-R-80ct-R;

R-80V-R-dele-R-80tm-R-86ct-R;

R-80V-R-dele-R-86tm-R-80ct-R;

R-86V-R-dele-R-80tm-R-80ct-R;

R-86V-R-dele-R-80tm-R-86ct-R;

R-86V-R-dele-R-86tm-R-80ct-R;

R-80V-R-dele-R-86tm-R-86ct-R;

R-80V-R-86C-R-80tm-R-80ct-R;

R-80V-R-86C-R-80tm-R-86ct-R;

R-80V-R-86C-R-86tm-R-80ct-R;

R-86V-R-86C-R-80tm-R-80ct-R;

R-86V-R-86C-R-80tm-R-86ct-R;

R-86V-R-86C-R-86tm-R-80ct-R;

R-80V-R-86C-R-86tm-R-86ct-R;

R-80V-R-dele-R-80tm-R-dele-R;

R-80V-R-dele-R-86tm-R-dele-R;

R-86V-R-dele-R-80tm-R-dele-R;

R-80V-R-86C-R-80tm-R-dele-R;

R-80V-R-86C-R-86tm-R-dele-R;

R-86V-R-86C-R-80tm-R-dele-R;

R-86V-R-86C-R-80tm-R-dele-R;

R-86V-R-86C-R-dele-R-80ct-R;

R-80V-R-86C-R-dele-R-80ct-R;

R-80V-R-dele-R-dele-R-80ct-R;

R-86V-R-dele-R-dele-R-80ct-R;

R-80V-R-86C-R-dele-R-dele-R; and

R- 80V-R; wherein

80V is the variable domain of CD80 or a functional fragment thereof;

86V is the variable domain of CD86 or a functional fragment thereof;

86C is the C domain of CD86 or a functional fragment thereof;

80tm is the transmembrane region of CD80 or a functional fragment thereof;

86tm is the transmembrane region of CD86 or a functional fragment thereof;

80ct is the cytoplasmic tail of CD80 or a functional fragment thereof;

86ct is the cytoplasmic tail of CD86 or a functional fragment thereof;

dele is 0 amino acids; and

R are each independently 0-100 amino acids.

In some embodiments, R are each independently 0-50 amino acids.

In some embodiments, R are each independently 0-30 amino acids.

In some embodiments, R are each independently 0-20 amino acids.

In some embodiments of the invention, the CD80 mutant is selected from the group consisting of:

CD80 with the C domain deleted;

CD80 with the C domain deleted and a CD86 transmembrane region substituting the CD80 transmembrane region;

CD80 with the C domain deleted and a CD86 cytoplasmic tail region substituting the CD80 cytoplasmic tail region;

CD80 with the C domain deleted and a CD86 V domain substituting the CD80 V domain;

CD80 with the C domain deleted and a CD86 V domain substituting the CD80 V domain and a CD86 transmembrane region substituting the CD80 transmembrane region;

CD80 with the C domain deleted and a CD86 V domain substituting the CD80 V domain and a CD86 cytoplasmic tail region substituting the CD80 cytoplasmic tail region;

CD80 with the C domain deleted and a CD86 transmembrane region substituting the CD80 transmembrane region and a CD86 cytoplasmic tail region substituting the CD80 cytoplasmic tail region;

CD80 with a CD86 C domain substituting the CD80 C domain;

CD80 with a CD86 C domain substituting the CD80 C domain and a CD86 transmembrane region substituting the CD80 transmembrane region;

CD80 with a CD86 C domain substituting the CD80 C domain and a CD86 cytoplasmic tail region substituting the CD80 cytoplasmic tail region;

CD80 with a CD86 C domain substituting the CD80 C domain and a CD86 V domain substituting the CD80 V domain;

CD80 with a CD86 C domain substituting the CD80 C domain and a CD86 V domain substituting the CD80 V domain and a CD86 transmembrane region substituting the CD80 transmembrane region;

CD80 with a CD86 C domain substituting the CD80 C domain and a CD86 V domain substituting the CD80 V domain and a CD86 cytoplasmic tail region substituting the CD80 cytoplasmic tail region;

CD80 with a CD86 C domain substituting the CD80 C domain and a CD86 transmembrane region substituting the CD80 transmembrane region and a CD86 cytoplasmic tail region substituting the CD80 cytoplasmic tail region;

CD80 with the C domain deleted and the cytoplasmic tail region deleted;

CD80 with the C domain deleted and the cytoplasmic tail region deleted and a CD86 transmembrane region substituting the CD80 transmembrane region;

CD80 with the C domain deleted and the cytoplasmic tail region deleted and a CD86 V domain substituting the CD80 V domain;

CD80 with a CD86 C domain substituting the CD80 C domain and a CD86 transmembrane region substituting the CD80 transmembrane region and a CD86 cytoplasmic tail region substituting the CD80 cytoplasmic tail region;

CD80 with a CD86 C domain substituting the CD80 C domain and the cytoplasmic tail region deleted;

CD80 with a CD86 C domain substituting the CD80 C domain and the cytoplasmic tail region deleted and a CD86 transmembrane region substituting the CD80 transmembrane region;

CD80 with a CD86 V domain substituting the CD80 V domain and a CD86 C domain substituting the CD80 C domain and the cytoplasmic tail region deleted;

CD80 with a CD86 V domain substituting the CD80 V domain and a CD86 C domain substituting the CD80 C domain and the transmembrane region deleted;

CD80 with a CD86 C domain substituting the CD80 C domain and the transmembrane region deleted;

CD80 with the C domain deleted and the transmembrane region deleted;

CD80 with the C domain deleted and CD86 V domain substituting the CD80 V domain and the transmembrane region deleted;

CD80 with a CD86 C domain substituting the CD80 C domain and the transmembrane region deleted and the cytoplasmic tail region deleted;

CD80 with the domain deleted, the transmembrane region deleted and the cytoplasmic tail region deleted; and

the CD80 variable domain or functional fragments thereof.

Protein forms of the CD80ΔC mutants can be formulated as components in vaccines or genetic constructs which include coding sequences that encode the CD80ΔC mutants may be provided as components of vaccines. In either case, such vaccines may be used in prophylactic or therapeutic methods.

According to some preferred embodiments of the invention, DNA vaccines are provided which contain DNA molecules that contain coding sequences encoding an immunogen and a CD80ΔC mutant. An improvement of the present invention relates to the inclusion of genetic material for the co-production of a CD80ΔC mutant protein in addition

to the production of the antigenic target encoded by nucleic acid sequences of the DNA vaccines.

The present invention relates to methods of introducing genetic material into the cells of an individual in order to induce immune responses against proteins and peptides which are encoded by the genetic material. The methods comprise the steps of administering to the tissue of said individual, either a single nucleic acid molecule that comprises a nucleotide sequence that encodes a target protein and a nucleotide sequence that encodes a CD80ΔC mutant protein; or a composition having two nucleic acid molecules, one that comprises a nucleotide sequence that encodes a target protein and one that comprises a nucleotide sequence that encodes a CD80ΔC mutant protein. The nucleic acid molecule(s) may be provided as plasmid DNA, the nucleic acid molecules of recombinant vectors or as part of the genetic material provided in an attenuated vaccine.

According to the present invention, compositions and methods are provided which prophylactically and/or therapeutically immunize an individual against a pathogen or abnormal, disease-related cell. The genetic material that encodes a target protein, i.e. a peptide or protein that shares at least an epitope with an immunogenic protein found on the pathogen or cells to be targeted, and genetic material that encodes a CD80ΔC mutant protein. The genetic material is expressed by the individual's cells and serves as an immunogenic target against which an immune response is elicited. The resulting immune response reacts with a pathogen or cells to be targeted and is broad based: in addition to a humoral immune response, both arms of the cellular immune response are elicited. The methods of the present invention are useful for conferring prophylactic and therapeutic immunity. Thus, a method of immunizing includes both methods of protecting an individual from pathogen challenge, or occurrence or proliferation of specific cells as well as methods of treating an individual suffering from pathogen infection, hyperproliferative disease or autoimmune disease.

As used herein the terms "target protein" and "immunogen" are used interchangeably and are meant to refer to peptides and protein encoded by gene constructs which act as protein targets for an immune response. The target protein is a protein against which an immune response can be elicited. The target protein is an immunogenic protein which shares at least an epitope with a protein from the pathogen or undesirable cell-type,

such as a cancer cell or a cell involved in autoimmune disease, against which immunization is required. The immune response directed against the target protein will protect the individual against and treat the individual for the specific infection or disease with which the target protein is associated. The target protein does not need to be identical to the protein against which an immune response is desired. Rather, the target protein must be capable of inducing an immune response that cross reacts to the protein against which the immune response is desired.

The present invention is useful to elicit broad immune responses against a target protein, i.e. proteins specifically associated with pathogens or the individual's own "abnormal" cells. The present invention is useful to immunize individuals against pathogenic agents and organisms such that an immune response against a pathogen protein provides protective immunity against the pathogen. The present invention is useful to combat hyperproliferative diseases and disorders such as cancer by eliciting an immune response against a target protein that is specifically associated with the hyperproliferative cells. The present invention is useful to combat autoimmune diseases and disorders by eliciting an immune response against a target protein that is specifically associated with cells involved in the autoimmune condition.

According to the present invention, DNA or RNA that encodes a target protein and a CD80ΔC mutant protein is introduced into the cells of tissue of an individual where it is expressed, thus producing the target protein and the CD80ΔC mutant protein. The DNA or RNA sequences encoding the target protein and the CD80ΔC mutant are each linked to regulatory elements necessary for expression in the cells of the individual. Regulatory elements for DNA expression include a promoter and a polyadenylation signal. In addition, other elements, such as a Kozak region, may also be included in the genetic construct. The preferred embodiments include nucleotide sequences encoding the target protein and CD80ΔC mutant protein provided as separate expressible forms in which each of the target protein and CD80ΔC mutant protein is linked to its own set of regulatory elements necessary for expression in the cell of the individual. However, the present invention additional relates to embodiments in which the target protein and CD80ΔC mutant protein are provided as a single genetic construct. In some such embodiment, the polyprotein which is produced by the single expressible form may be processed into two

separate proteins or it may exist as a chimeric protein which functions both as the target protein and CD80ΔC mutant. In some embodiments, nucleic acid sequences encoding two or more copies of the target protein and/or two or more copies CD80ΔC mutant protein may be provided in a single expressible form of a gene construct. Polyproteins encoded therein may be processed into subunits following expression or maintained as functional polyproteins.

As used herein, the term "expressible form" refers to gene constructs which contain the necessary regulatory elements operable linked to a coding sequence that encodes a target protein and/or CD80ΔC mutant protein, such that when present in the cell of the individual, the coding sequence will be expressed.

As used herein, the term "sharing an epitope" refers to proteins which comprise at least one epitope that is identical to or substantially similar to an epitope of another protein.

As used herein, the term "substantially similar epitope" is meant to refer to an epitope that has a structure which is not identical to an epitope of a protein but nonetheless invokes an cellular or humoral immune response which cross reacts to that protein.

Genetic constructs comprise a nucleotide sequence that encodes a target protein and/or a CD80ΔC mutant protein operably linked to regulatory elements needed for gene expression. According to the invention, combinations of gene constructs which include one that comprises an expressible form of the nucleotide sequence that encodes a target protein and one that includes an expressible form of the nucleotide sequence that encodes a CD80ΔC mutant protein are provided. Incorporation into a living cell of the DNA or RNA molecule(s) which include the combination of gene constructs results in the expression of the DNA or RNA and production of the target protein and a CD80ΔC mutant protein. A surprisingly enhanced immune response against the target protein results.

The present invention may be used to immunize an individual against all pathogens such as viruses, prokaryote and pathogenic eukaryotic organisms such as unicellular pathogenic organisms and multicellular parasites. The present invention is particularly useful to immunize an individual against those pathogens which infect cells and which are not encapsulated such as viruses, and prokaryote such as gonorrhea, listeria and

shigella. In addition, the present invention is also useful to immunize an individual against protozoan pathogens which include a stage in the life cycle where they are intracellular pathogens. As used herein, the term "intracellular pathogen" is meant to refer to a virus or pathogenic organism that, at least part of its reproductive or life cycle, exists within a host cell and therein produces or causes to be produced, pathogen proteins. Table 1 provides a listing of some of the viral families and genera for which vaccines according to the present invention can be made. DNA constructs that comprise DNA sequences which encode the peptides that comprise at least an epitope identical or substantially similar to an epitope displayed on a pathogen antigen such as those antigens listed on the tables are useful in vaccines. Moreover, the present invention is also useful to immunize an individual against other pathogens including prokaryotic and eukaryotic protozoan pathogens as well as multicellular parasites such as those listed on Table 2.

In order to produce a genetic vaccine to protect against pathogen infection, genetic material which encodes immunogenic proteins against which a protective immune response can be mounted must be included in a genetic construct as the coding sequence for the target. Whether the pathogen infects intracellularly, for which the present invention is particularly useful, or extracellularly, it is unlikely that all pathogen antigens will elicit a protective response. Because DNA and RNA are both relatively small and can be produced relatively easily, the present invention provides the additional advantage of allowing for vaccination with multiple pathogen antigens. The genetic construct used in the genetic vaccine can include genetic material which encodes many pathogen antigens. For example, several viral genes may be included in a single construct thereby providing multiple targets.

Tables 1 and 2 include lists of some of the pathogenic agents and organisms for which genetic vaccines can be prepared to protect an individual from infection by them. In some preferred embodiments, the methods of immunizing an individual against a pathogen are directed against HIV, HTLV or HBV.

Another aspect of the present invention provides a method of conferring a broad based protective immune response against hyperproliferating cells that are characteristic in hyperproliferative diseases and to a method of treating individuals suffering from hyperproliferative diseases. As used herein, the term "hyperproliferative diseases" is

meant to refer to those diseases and disorders characterized by hyperproliferation of cells. Examples of hyperproliferative diseases include all forms of cancer and psoriasis.

It has been discovered that introduction of a genetic construct that includes a nucleotide sequence which encodes an immunogenic "hyperproliferating cell"-associated protein into the cells of an individual results in the production of those proteins in the vaccinated cells of an individual. As used herein, the term "hyperproliferative-associated protein" is meant to refer to proteins that are associated with a hyperproliferative disease. To immunize against hyperproliferative diseases, a genetic construct that includes a nucleotide sequence which encodes a protein that is associated with a hyperproliferative disease is administered to an individual.

In order for the hyperproliferative-associated protein to be an effective immunogenic target, it must be a protein that is produced exclusively or at higher levels in hyperproliferative cells as compared to normal cells. Target antigens include such proteins, fragments thereof and peptides which comprise at least an epitope found on such proteins. In some cases, a hyperproliferative-associated protein is the product of a mutation of a gene that encodes a protein. The mutated gene encodes a protein which is nearly identical to the normal protein except it has a slightly different amino acid sequence which results in a different epitope not found on the normal protein. Such target proteins include those which are proteins encoded by oncogenes such as *myb, myc, fyn,* and the translocation gene *bcr/abl, ras, src,* P53, *neu, trk* and EGRF. In addition to oncogene products as target antigens, target proteins for anti-cancer treatments and protective regimens include variable regions of antibodies made by B cell lymphomas and variable regions of T cell receptors of T cell lymphomas which, in some embodiments, are also used target antigens for autoimmune disease. Other tumor-associated proteins can be used as target proteins such as proteins which are found at higher levels in tumor cells including the protein recognized by monoclonal antibody 17-1A and folate binding proteins.

While the present invention may be used to immunize an individual against one or more of several forms of cancer, the present invention is particularly useful to prophylactically immunize an individual who is predisposed to develop a particular cancer or who has had cancer and is therefore susceptible to a relapse. Developments in genetics and technology as well as epidemiology allow for the determination of probability and risk

assessment for the development of cancer in individual. Using genetic screening and/or family health histories, it is possible to predict the probability a particular individual has for developing any one of several types of cancer.

Similarly, those individuals who have already developed cancer and who have been treated to remove the cancer or are otherwise in remission are particularly susceptible to relapse and reoccurrence. As part of a treatment regimen, such individuals can be immunized against the cancer that they have been diagnosed as having had in order to combat a recurrence. Thus, once it is known that an individual has had a type of cancer and is at risk of a relapse, they can be immunized in order to prepare their immune system to combat any future appearance of the cancer.

The present invention provides a method of treating individuals suffering from hyperproliferative diseases. In such methods, the introduction of genetic constructs serves as an immunotherapeutic, directing and promoting the immune system of the individual to combat hyperproliferative cells that produce the target protein.

The present invention provides a method of treating individuals suffering from autoimmune diseases and disorders by conferring a broad based protective immune response against targets that are associated with autoimmunity including cell receptors and cells which produce "self"-directed antibodies.

T cell mediated autoimmune diseases include Rheumatoid arthritis (RA), multiple sclerosis (MS), Sjogren's syndrome, sarcoidosis, insulin dependent diabetes mellitus (IDDM), autoimmune thyroiditis, reactive arthritis, ankylosing spondylitis, scleroderma, polymyositis, dermatomyositis, psoriasis, vasculitis, Wegener's granulomatosis, Crohn's disease and ulcerative colitis. Each of these diseases is characterized by T cell receptors that bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases. Vaccination against the variable region of the T cells would elicit an immune response including CTLs to eliminate those T cells.

In RA, several specific variable regions of T cell receptors (TCRs) which are involved in the disease have been characterized. These TCRs include Vβ-3, Vβ-14, Vβ-17 and Vα-17. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in RA. See: Howell,

M.D., *et al.*, 1991 *Proc. Natl. Acad. Sci. USA* **88**:10921-10925; Paliard, X., *et al.*, 1991 *Science* **253**:325-329; Williams, W.V., *et al.*, 1992 *J. Clin. Invest.* **90**:326-333; each of which is incorporated herein by reference.

In MS, several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include Vβ-7 and Vα-10. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in MS. See: Wucherpfennig, K.W., *et al.*, 1990 *Science* **248**:1016-1019; Oksenberg, J.R., *et al.*, 1990 *Nature* **345**:344-346; each of which is incorporated herein by reference.

In scleroderma, several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include Vβ-6, Vβ-8, Vβ-14 and Vα-16, Vα-3C, Vα-7, Vα-14, Vα-15, Vα-16, Vα-28 and Vα-12. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in scleroderma.

In order to treat patients suffering from a T cell mediated autoimmune disease, particularly those for which the variable region of the TCR has yet to be characterized, a synovial biopsy can be performed. Samples of the T cells present can be taken and the variable region of those TCRs identified using standard techniques. Genetic vaccines can be prepared using this information.

B cell mediated autoimmune diseases include Lupus (SLE), Grave's disease, myasthenia gravis, autoimmune hemolytic anemia, autoimmune thrombocytopenia, asthma, cryoglobulinemia, primary biliary sclerosis and pernicious anemia. Each of these diseases is characterized by antibodies which bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases. Vaccination against the variable region of antibodies would elicit an immune response including CTLs to eliminate those B cells that produce the antibody.

In order to treat patients suffering from a B cell mediated autoimmune disease, the variable region of the antibodies involved in the autoimmune activity must be identified. A biopsy can be performed and samples of the antibodies present at a site of inflammation can be taken. The variable region of those antibodies can be identified using standard techniques. Genetic vaccines can be prepared using this information.

In the case of SLE, one antigen is believed to be DNA. Thus, in patients to be immunized against SLE, their sera can be screened for anti-DNA antibodies and a vaccine can be prepared which includes DNA constructs that encode the variable region of such anti-DNA antibodies found in the sera.

Common structural features among the variable regions of both TCRs and antibodies are well known. The DNA sequence encoding a particular TCR or antibody can generally be found following well known methods such as those described in Kabat, *et al.* 1987 *Sequence of Proteins of Immunological Interest* U.S. Department of Health and Human Services, Bethesda MD, which is incorporated herein by reference. In addition, a general method for cloning functional variable regions from antibodies can be found in Chaudhary, V.K., *et al.*, 1990 *Proc. Natl. Acad. Sci. USA* **87**:1066, which is incorporated herein by reference.

The present invention provides an improved method of immunizing individuals that comprises the step of delivering gene constructs to the cells of individuals as part of vaccine compositions which include are provided which include DNA vaccines, attenuated live vaccines and recombinant vaccines. The gene constructs comprise a nucleotide sequence that encodes an immunomodulating protein and that is operably linked to regulatory sequences that can function in the vaccinee to effect expression. The improved vaccines result in an enhanced cellular immune response.

In some methods of immunizing, the individual is administered a gene construct encoding an immunogen and a genetic construct encoding a CD80ΔC mutant protein. In some methods of immunizing, the individual is administered a gene construct encoding both an immunogen and a CD80ΔC mutant protein. In some alternative methods of immunizing, the individual is administered an immunogen and a CD80ΔC mutant protein. In some alternative methods of immunizing, the individual is administered a protein immunogen and genetic construct encoding a CD80ΔC mutant protein. In some alternative methods of immunizing, the individual is administered a gene construct encoding an immunogen and a CD80ΔC mutant protein.

According to another aspect of the invention, CD80 C region proteins are provided to suppress immune responses associated with autoimmune diseases and transplant rejections. The CD80 C region proteins contain a functional CD80 C region. Functional

fragments of the CD80 C region can be identified routinely. In some embodiments, functional fragments of the CD80 C region are less than 60 amino acids. In some embodiments, functional fragments of the CD80 C region are less than 50 amino acids. In some embodiments, functional fragments of the CD80 C region are less than 40 amino acids. In some embodiments, functional fragments of the CD80 C region are less than 30 amino acids. In some embodiments, functional fragments of the CD80 C region are less than 20 amino acids. In some embodiments, functional fragments of the CD80 C region are less than 15 amino acids. In some embodiments, functional fragments of the CD80 C region are less than 10 amino acids.

In some embodiments, the V region is deleted. In some embodiments, the CD80 or CD86 V region is present. Some embodiments include the CD80 transmembrane region. Some embodiments include the CD86 transmembrane region. In some embodiments, the CD80 transmembrane region is deleted and not substituted by any other sequences. Some embodiments include non-CD-80, non-CD86 sequences. Some embodiments include non-CD-80, non-CD86 sequences in place of the CD80tm. Some embodiments include the CD80 cytoplasmic tail. Some embodiments include the CD86 cytoplasmic tail. In some embodiments, the CD80 cytoplasmic tail is deleted and not substituted by any other sequences. Some embodiments include non-CD-80, non-CD86 sequences in place of the CD80 ct.

According to some embodiments, the non-CD80 protein comprises at least the C domain of CD80 or a functional fragment thereof. As used herein, the term non-CD80 protein is meant to refer to a protein which differs from wildtype CD80 but comprises a CD80 C domain or a functional fragment thereof. In some embodiments, the non-CD80 protein have the formula:

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8\text{-}R^9$$

wherein

R^1 is 0-50 amino acids;

R^2 is 80V or 86V;

R^3 is 0-50 amino acids;

R^4 is 80C;

R^5 is 0-50 amino acids;

R^6 is 80tm or 86tm;

R^7 is 0-50 amino acids;

R^8 is 80ct or 86ct; and

R^9 is 0-50 amino acids

wherein

80V is the variable domain of CD80 or a functional fragment thereof;

86V is the variable domain of CD86 or a functional fragment thereof;

80C is the C domain of CD80 or a functional fragment thereof;

80tm is the transmembrane region of CD80 or a functional fragment thereof;

86tm is the transmembrane region of CD86 or a functional fragment thereof;

80ct is the cytoplasmic tail of CD80 or a functional fragment thereof; and

86ct is the cytoplasmic tail of CD86 or a functional fragment thereof.

According to some embodiments of the invention, the isolated non-CD80 protein that comprises at least the C domain of CD80 or a functional fragment thereof has the formula selected from the group consisting of:

R-dele-R-80C-R-80tm-R-80ct-R;

R-dele-R-80C-R-80tm-R-dele-R;

R-80V-R-80C-R-80tm-R-dele-R;

R-80V-R-80C-R-dele-R-dele-R;

R-86V-R-80C-R-80tm-R-80ct-R;

R-86V-R-80C-R-80tm-R-dele-R;

R-86V-R-80C-R-dele-R-dele-R;

R-80V-R-80C-R-86tm-R-80ct-R;

R-dele-R-80C-R-86tm-R-80ct-R;

R-dele-R-80C-R-86tm-R-dele-R;

R-80V-R-80C-R-86tm-R-dele-R;

R-80V-R-80C-R-80tm-R-86ct-R;

R-dele-R-80C-R-80tm-R-86ct-R;

R-86V-R-80C-R-86tm-R-80ct-R;

R-86V-R-80C-R-80tm-R-86ct-R;

R-86V-R-80C-R-86tm-R-dele-R;

R-dele-R-80C-R-86tm-R-86ct-R; and

R-86V-R-80C-R-86tm-R-86ct;

wherein

80V is the variable domain of CD80 or a functional fragment thereof;

86V is the variable domain of CD86 or a functional fragment thereof;

80C is the C domain of CD80 or a functional fragment thereof;

80tm is the transmembrane region of CD80 or a functional fragment thereof;

86tm is the transmembrane region of CD86 or a functional fragment thereof;

80ct is the cytoplasmic tail of CD80 or a functional fragment thereof;

86ct is the cytoplasmic tail of CD86 or a functional fragment thereof;

dele is 0 amino acids; and

each R is each independently 0-100 amino acids.

In some embodiments, each R is independently 0-50 amino acids; in some embodiments, each R is independently 0-30 amino acids; in some embodiments, each R is independently 0-20 amino acids.

In some embodiments of the invention, the non-CD80 protein is selected from the group consisting of:

a mutant CD80 with the variable domain deleted;

a mutant CD80 with the variable domain deleted and the cytoplasmic tail deleted;

a mutant CD80 with the cytoplasmic tail deleted;

a mutant CD80 with the transmembrane region deleted and the cytoplasmic tail deleted;

a mutant CD80 with a CD86 variable domain substituted in place of the CD80 variable domain;

a mutant CD80 with a CD86 variable domain substituted in place of the CD80 variable domain and the cytoplasmic tail deleted;

a mutant CD80 with a CD86 variable domain substituted in place of the CD80 variable domain and the transmembrane region deleted and the cytoplasmic tail deleted;

a mutant CD80 with a CD86 transmembrane region substituted in place of the CD80 transmembrane region;

a mutant CD80 with the variable region deleted and a CD86 transmembrane region substituted in place of the CD80 transmembrane region;

a mutant CD80 with the variable region deleted, the cytoplasmic tail deleted and a CD86 transmembrane region substituted in place of the CD80 transmembrane region;

a mutant CD80 with the cytoplasmic tail deleted and a CD86 transmembrane region substituted in place of the CD80 transmembrane region;

a mutant CD80 with a CD86 cytoplasmic tail substituted in place of the CD80 cytoplasmic tail;

a mutant CD80 with the variable region deleted and a CD86 cytoplasmic tail substituted in place of the CD80 cytoplasmic tail;

a mutant CD80 with a CD86 variable domain substituted in place of the CD80 variable domain and a CD86 transmembrane region substituted in place of the CD80 transmembrane region;

a mutant CD80 with a CD86 variable domain substituted in place of the CD80 variable domain and a CD86 cytoplasmic tail substituted in place of the CD80 cytoplasmic tail;

a mutant CD80 with a CD86 variable domain substituted in place of the CD80 variable domain and a CD86 transmembrane region substituted in place of the CD80 transmembrane region and the cytoplasmic tail deleted;

a mutant CD80 with the variable domain deleted and a CD86 transmembrane region substituted in place of the CD80 transmembrane region and CD86 cytoplasmic tail substituted in place of the CD80 cytoplasmic tail; and

a mutant CD80 with a CD86 variable domain substituted in place of the CD80 variable domain and a CD86 transmembrane region substituted in place of the CD80

transmembrane region and CD86 cytoplasmic tail substituted in place of the CD80 cytoplasmic tail.

The CD80 C region proteins are provided as either proteins or genetic constructs that encode CD80 C region. Delivery of genetic constructs which comprise coding sequences that encode the wild type CD80, dele/80C/80tm/80ct, dele/80C/80tm/86ct dele/80C/86tm/80ct or dele/80C/86tm/86ct provide particularly effective results in immunosuppression and treatment of autoimmune diseases.

The methods of this aspect of the invention are useful to treat autoimmune diseases and disorders. Those skilled in the art can identify individuals who have autoimmune diseases or disorders. Examples of autoimmune diseases and disorders include T cell mediated autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), Sjogren's syndrome, sarcoidosis, insulin dependent diabetes mellitus (IDDM), autoimmune thyroiditis, reactive arthritis, ankylosing spondylitis, scleroderma, polymyositis, dermatomyositis, psoriasis, vasculitis, Wegener's granulomatosis, Crohn's disease and ulcerative colitis, and B cell mediated autoimmune diseases such as Lupus (SLE), Grave's disease, myasthenia gravis, autoimmune hemolytic anemia, autoimmune thrombocytopenia, asthma, cryoglobulinemia, primary biliary sclerosis and pernicious anemia.

The methods of this aspect of the invention are also useful to suppress immune responses in individuals undergoing transplant procedures including cell transplants such as bone marrow and brain cell grafts, tissue transplants such as cornea and skin grafts and myoplasty procedures, and organ transplants such as liver, lung, kidney and heart.

The methods of making and delivering compositions of the present invention are generally the same for immunization protocols as well as non-immunogenic therapeutic protocols.

As used herein, the term "protein" is meant to include proteinaceous molecules including peptides, polypeptides and proteins. Some embodiments of the invention relate to delivery of proteins through the administration of nucleic acids, particularly DNA, and to methods of using the same. For example, in some methods of immunizing, nucleic acids that encode immunogenic proteins and CD80ΔC mutant proteins are administered to individuals. Likewise, in some methods of treating autoimmune diseases

and preventing graft/transplant rejections by immunosuppression, nucleic acids that encode CD80 C region proteins are administered to individuals. As used herein, the term "gene constructs of the invention" is intended to mean gene constructs that include coding sequences which encode immunogenic proteins, CD80ΔC mutant proteins and CD80 C region proteins which each can be produced by similar means and which can be formulated and administered in a similar manner for use in methods of the invention.

DNA vaccines are described in U.S. Patent No. 5,593,972, U.S. Patent No. 5,589,466, PCT/US90/01515, PCT/US93/02338, PCT/US93/048131, and PCT/US94/00899, which are each incorporated herein by reference. In addition to the delivery protocols described in those applications, alternative methods of delivering DNA are described in U.S. Patent Nos. 4,945,050 and 5,036,006, which are both incorporated herein by reference. DNA vaccine protocols are useful to immunize individuals. The teachings can be applied in the present invention to aspects in which individuals with autoimmune disease and transplant rejections are treated using gene constructs that encode CD80 C region proteins. In such embodiments, no coding sequences encoding immunogens are provided.

When taken up by a cell, the genetic constructs of the invention may remain present in the cell as a functioning extrachromosomal molecule and/or integrate into the cell's chromosomal DNA. DNA may be introduced into cells where it remains as separate genetic material in the form of a plasmid or plasmids. Alternatively, linear DNA which can integrate into the chromosome may be introduced into the cell. When introducing DNA into the cell, reagents which promote DNA integration into chromosomes may be added. DNA sequences which are useful to promote integration may also be included in the DNA molecule. Alternatively, RNA may be administered to the cell. It is also contemplated to provide the genetic constructs of the invention as a linear minichromosome including a centromere, telomeres and an origin of replication.

Genetic constructs of the invention include regulatory elements necessary for gene expression of a nucleic acid molecule. The elements include: a promoter, an initiation codon, a stop codon, and a polyadenylation signal. In addition, enhancers are often required for gene expression of the sequence that encodes the protein of the invention. It is necessary that these elements be operable linked to the sequence that encodes the desired proteins and that the regulatory elements are operably in the individual to whom they are administered.

Initiation codons and stop codon are generally considered to be part of a nucleotide sequence that encodes the desired protein. However, it is necessary that these elements are functional in the individual to whom the gene construct is administered. The initiation and termination codons must be in frame with the coding sequence.

Promoters and polyadenylation signals used must be functional within the cells of the individual.

Examples of promoters useful to practice the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to promoters from Simian Virus 40 (SV40), Mouse Mammary Tumor Virus (MMTV) promoter, Human Immunodeficiency Virus (HIV) such as the HIV Long Terminal Repeat (LTR) promoter, Moloney virus, ALV, Cytomegalovirus (CMV) such as the CMV immediate early promoter, Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV) as well as promoters from human genes such as human Actin, human Myosin, human Hemoglobin, human muscle creatine and human metalothionein.

Examples of polyadenylation signals useful to practice the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to human and bovine growth hormone polyadenylation signals, SV40 polyadenylation signals and LTR polyadenylation signals. In particular, the SV40 polyadenylation signal which is in pCEP4 plasmid (Invitrogen, San Diego CA), referred to as the SV40 polyadenylation signal, is used.

In addition to the regulatory elements required for DNA expression, other elements may also be included in the DNA molecule. Such additional elements include enhancers. The enhancer may be selected from the group including but not limited to: human Actin, human Myosin, human Hemoglobin, human muscle creatine and viral enhancers such as those from CMV, RSV and EBV.

Genetic constructs of the invention can be provided with mammalian origin of replication in order to maintain the construct extrachromosomally and produce multiple copies of the construct in the cell. Plasmids pCEP4 and pREP4 from Invitrogen (San Diego, CA) contain the Epstein Barr virus origin of replication and nuclear antigen EBNA-1 coding region which produces high copy episomal replication without integration.

In some preferred embodiments related to immunization applications, nucleic acid molecule(s) are delivered which include nucleotide sequences that encode a target protein, a CD80ΔC mutant protein and, additionally, genes for proteins which further enhance the immune response against such target proteins. Examples of such genes are those which encode cytokines and lymphokines such as α-interferon, gamma-interferon, platelet derived growth factor (PDGF), GC-SF, GM-CSF, TNF, epidermal growth factor (EGF), IL-1, IL-2, IL-4, IL-6, IL-8, IL-10 and IL-12.

In order to maximize protein production, regulatory sequences may be selected which are well suited for gene expression in the cells the construct is administered into. Moreover, codons may be selected which are most efficiently transcribed in the cell. One having ordinary skill in the art can produce DNA constructs which are functional in the cells.

The methods of the present invention, whether methods of immunizing or methods of immunosuppressing, comprise the step of administering nucleic acid molecules to tissue of the individual. In some preferred embodiments, the nucleic acid molecules are administered intramuscularly, intranasally, intraperatoneally, subcutaneously, intradermally, intravenously, by aerosol administration to lung tissue or topically or by lavage to mucosal tissue selected from the group consisting of vaginal, rectal, urethral, buccal and sublingual.

An aspect of the present invention relates to pharmaceutical compositions useful in the methods of the present invention. The pharmaceutical compositions comprise a nucleic acid molecule, preferably a DNA molecule comprising a nucleotide sequence that encodes one or more proteins operably linked to regulatory elements necessary for expression in the cells of the individual. The pharmaceutical compositions further comprise a pharmaceutically acceptable carrier or diluent. The term "pharmaceutical" is well known and widely understood by those skilled in the art. As used herein, the terms "pharmaceutical compositions" and "injectable pharmaceutical compositions" are meant to have their ordinary meaning as understood by those skilled in the art. Pharmaceutical compositions are required to meet specific standards regarding sterility, pyrogens, particulate matter as well as isotonicity and pH. For example, injectable pharmaceuticals are sterile and pyrogen free.

Pharmaceutical compositions according to the present invention may comprise about 1 ng to about 10,000 μg of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 2000 μg, 3000 μg, 4000 μg or 5000 μg of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 1000 μg of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 10 ng to about 800 μg of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 0.1 to about 500 μg of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 1 to about 350 μg of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 25 to about 250 μg of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 100 μg DNA.

The pharmaceutical compositions according to the present invention which comprise gene constructs of the invention are formulated according to the mode of administration to be used. One having ordinary skill in the art can readily formulate a vaccine or non-immunogenic therapeutic that comprises a genetic construct. In cases where intramuscular injection is the chosen mode of administration, an isotonic formulation is preferably used. Generally, additives for isotonicity can include sodium chloride, dextrose, mannitol, sorbitol and lactose. In some cases, isotonic solutions such as phosphate buffered saline are preferred. Stabilizers include gelatin and albumin. In some embodiments, a vasoconstriction agent is added to the formulation. The pharmaceutical preparations according to the present invention are provided sterile and pyrogen free. Pharmaceutical compositions according to the invention include delivery components in combination with nucleic acid molecules which further comprise a pharmaceutically acceptable carriers or vehicles, such as, for example, saline. Any medium may be used which allows for successful delivery of the nucleic acid. One skilled in the art would readily comprehend the multitude of pharmaceutically acceptable media that may be used in the present invention. Suitable pharmaceutical carriers are described in *Remington's Pharmaceutical Sciences*, A. Osol, a standard reference text in this field, which is incorporated herein by reference.

In some embodiments, the nucleic acid molecule is delivered to the cells in conjunction with administration of a facilitating agent. Facilitating agents are also referred to as polynucleotide function enhancers or genetic vaccine facilitator agents. Facilitating

agents are described in U.S. Patent No. 5,830,876 issued November 3, 1998, U.S. Patent Number 5,593,972 issued January 14, 1997 and International Application Serial Number PCT/US94/00899 filed January 26, 1994 (U.S. Serial No. 08/979,385 filed November 29, 1997), which are each incorporated herein by reference. In addition, facilitating agents are described in U.S. 5,739,118 issued April 14, 1998, U.S. Patent No. 5,837,533 issued November 17, 1998, PCT/US95/12502 filed September 28, 1995 and PCT/US95/04071 filed March 30, 1995, which are each incorporated herein by reference. Facilitating agents which are administered in conjunction with nucleic acid molecules may be administered as a mixture with the nucleic acid molecule or administered separately simultaneously, before or after administration of nucleic acid molecules. In addition, other agents which may function transfecting agents and/or replicating agents and/or inflammatory agents and which may be co-administered with or without a facilitating agent include growth factors, cytokines and lymphokines such as α-interferon, gamma-interferon, platelet derived growth factor (PDGF), GC-SF, GM-CSF, TNF, epidermal growth factor (EGF), IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12 and B7.2 as well as fibroblast growth factor, surface active agents such as immune-stimulating complexes (ISCOMS), Freund's incomplete adjuvant, LPS analog including monophosphoryl Lipid A (MPL), muramyl peptides, quinone analogs and vesicles such as squalene and squalene, and hyaluronic acid. In embodiments which relate to methods of immunizing, co-agents are selected which preferably enhance immune responses. In embodiments which relate to methods of immunosuppressing, co-agents are selected which do not enhance immune responses.

In some preferred embodiments, the genetic constructs of the invention are formulated with or administered in conjunction with a facilitator selected from the group consisting of benzoic acid esters, anilides, amidines, urethans and the hydrochloride salts thereof such as those of the family of local anesthetics.

The facilitators in some preferred embodiments may be a compound having one of the following formulae:

$$Ar - R^1 - O - R^2 - R^3$$

or

$$Ar - N - R^1 - R^2 - R^3$$

or

$R^4 - N - R^5 - R^6$

or

$R^4 - O - R^1 - - R^7$

wherein:

Ar is benzene, *p*-aminobenzene, *m*-aminobenzene, *o*-aminobenzene, substituted benzene, substituted *p*-aminobenzene, substituted *m*-aminobenzene, substituted *o*-aminobenzene, wherein the amino group in the aminobenzene compounds can be amino, C_1-C_5 alkylamine, C_1-C_5, C_1-C_5 dialkylamine and substitutions in substituted compounds are halogen, C_1-C_5 alkyl and C_1-C_5 alkoxy;

R^1 is C=O;

R^2 is C_1-C_{10} alkyl including branched alkyls;

R^3 is hydrogen, amine, C_1-C_5 alkylamine, C_1-C_5, C_1-C_5 dialkylamine;

$R^2 + R^3$ can form a cyclic alkyl, a C_1-C_{10} alkyl substituted cyclic alkyl, a cyclic aliphatic amine, a C_1-C_{10} alkyl substituted cyclic aliphatic amine, a heterocycle, a C_1-C_{10} alkyl substituted heterocycle including a C_1-C_{10} alkyl N-substituted heterocycle;

R^4 is Ar, R^2 or C_1-C_5 alkoxy, a cyclic alkyl, a C_1-C_{10} alkyl substituted cyclic alkyl, a cyclic aliphatic amine, a C_1-C_{10} alkyl substituted cyclic aliphatic amine, a heterocycle, a C_1-C_{10} alkyl substituted heterocycle and a C_1-C_{10} alkoxy substituted heterocycle including a C_1-C_{10} alkyl N-substituted heterocycle;

R^5 is C=NH;

R^6 is Ar, R^2 or C_1-C_5 alkoxy, a cyclic alkyl, a C_1-C_{10} alkyl substituted cyclic alkyl, a cyclic aliphatic amine, a C_1-C_{10} alkyl substituted cyclic aliphatic amine, a heterocycle, a C_1-C_{10} alkyl substituted heterocycle and a C_1-C_{10} alkoxy substituted heterocycle including a C_1-C_{10} alkyl N-substituted heterocycle; and.

R^7 is Ar, R^2 or C_1-C_5 alkoxy, a cyclic alkyl, a C_1-C_{10} alkyl substituted cyclic alkyl, a cyclic aliphatic amine, a C_1-C_{10} alkyl substituted cyclic aliphatic amine, a heterocycle, a C_1-C_{10} alkyl substituted heterocycle and a C_1-C_{10} alkoxy substituted heterocycle including a C_1-C_{10} alkyl N-substituted heterocycle.

Examples of esters include: benzoic acid esters such as piperocaine, meprylcaine and isobucaine; *para*-aminobenzoic acid esters such as procaine, tetracaine,

butethamine, propoxycaine and chloroprocaine; *meta*-aminobenzoic acid esters including metabuthamine and primacaine; and *para*-ethoxybenzoic acid esters such as parethoxycaine. Examples of anilides include lidocaine, etidocaine, mepivacaine, bupivacaine, pyrrocaine and prilocaine. Other examples of such compounds include dibucaine, benzocaine, dyclonine, pramoxine, proparacaine, butacaine, benoxinate, carbocaine, methyl bupivacaine, butasin picrate, phenacaine, diothan, luccaine, intracaine, nupercaine, metabutoxycaine, piridocaine, biphenamine and the botanically-derived bicyclics such as cocaine, cinnamoylcocaine, truxilline and cocaethylene and all such compounds complexed with hydrochloride.

In preferred embodiments, the facilitator is bupivacaine. The difference between bupivacaine and mepivacaine is that bupivacaine has a N-butyl group in place of an N-methyl group of mepivacaine. Compounds may have at that N, C_1-C_{10}. Compounds may be substituted by halogen such as procaine and chloroprocaine. The anilides are preferred.

The facilitating agent is administered prior to, simultaneously with or subsequent to the genetic construct. The facilitating agent and the genetic construct may be formulated in the same composition.

Bupivacaine-HCl is chemically designated as 2-piperidinecarboxamide, 1-butyl-N-(2,6-dimethylphenyl)-monohydrochloride, monohydrate and is widely available commercially for pharmaceutical uses from many sources including from Astra Pharmaceutical Products Inc. (Westboro, MA) and Sanofi Winthrop Pharmaceuticals (New York, NY), Eastman Kodak (Rochester, NY). Bupivacaine is commercially formulated with and without methylparaben and with or without epinephrine. Any such formulation may be used. It is commercially available for pharmaceutical use in concentration of 0.25%, 0.5% and 0.75% which may be used on the invention. Alternative concentrations, particularly those between 0.05% - 1.0% which elicit desirable effects may be prepared if desired. According to the present invention, about 250 μg to about 10 mg of bupivacaine is administered. In some embodiments, about 250 μg to about 7.5 mg is administered. In some embodiments, about 0.05 mg to about 5.0 mg is administered. In some embodiments, about 0.5 mg to about 3.0 mg is administered. In some embodiments about 5 to 50 μg is administered. For example, in some embodiments about 50 μl to about 2 ml, preferably 50

μl to about 1500 μl and more preferably about 1 ml of 0.25-0.50% bupivacaine-HCl and 0.1% methylparaben in an isotonic pharmaceutical carrier is administered at the same site as the vaccine before, simultaneously with or after the vaccine is administered. Similarly, in some embodiments, about 50 μl to about 2 ml, preferably 50 μl to about 1500 μl and more preferably about 1 ml of 0.25-0.50% bupivacaine-HCl in an isotonic pharmaceutical carrier is administered at the same site as the vaccine before, simultaneously with or after the vaccine is administered. Bupivacaine and any other similarly acting compounds, particularly those of the related family of local anesthetics may be administered at concentrations which provide the desired facilitation of uptake of genetic constructs by cells.

In some embodiments of the invention, the individual is first subject to injection of the facilitator prior to administration of the genetic construct. That is, up to, for example, up to a about a week to ten days prior to administration of the genetic construct, the individual is first injected with the facilitator. In some embodiments, the individual is injected with facilitator about 1 to 5 days, in some embodiments 24 hours, before or after administration of the genetic construct. Alternatively, if used at all, the facilitator is administered simultaneously, minutes before or after administration of the genetic construct. Accordingly, the facilitator and the genetic construct may be combined to form a single pharmaceutical compositions.

In some embodiments, the genetic constructs are administered free of facilitating agents, that is in formulations free from facilitating agents using administration protocols in which the genetic constructions are not administered in conjunction with the administration of facilitating agents.

In some embodiments relating to immunization, gene constructs of the invention may remain part of the genetic material in attenuated live microorganisms or recombinant microbial vectors. In addition to using expressible forms of CD80 CD80ΔC mutant proteins coding sequences to improve genetic vaccines, the present invention relates to improved attenuated live vaccines and improved vaccines which use recombinant vectors to deliver foreign genes that encode antigens. Examples of attenuated live vaccines and those using recombinant vectors to deliver foreign antigens are described in U.S. Patent Nos.: 4,722,848; 5,017,487; 5,077,044; 5,110,587; 5,112,749; 5,174,993; 5,223,424; 5,225,336; 5,240,703; 5,242,829; 5,294,441; 5,294,548; 5,310,668; 5,387,744; 5,389,368;

5,424,065; 5,451,499; 5,453,364; 5,462,734; 5,470,734; and 5,482,713, which are each incorporated herein by reference. Gene constructs are provided which include the nucleotide sequence that encodes a CD80ΔC mutants protein is operably linked to regulatory sequences that can function in the vaccinee to effect expression. The gene constructs are incorporated in the attenuated live vaccines and recombinant vaccines to produce improved vaccines according to the invention. Gene constructs may be part of genomes of recombinant viral vaccines where the genetic material either integrates into the chromosome of the cell or remains extrachromosomal. In some embodiments relating to non-immune response inducing therapy, nucleic acid molecules that encode CD80 C region protein may be delivered using any one of a variety of delivery components, such as recombinant viral expression vectors or other suitable delivery means, so as to affect their introduction and expression in compatible host cells. In general, viral vectors may be DNA viruses such as recombinant adenoviruses and recombinant vaccinia viruses or RNA viruses such as recombinant retroviruses. Other recombinant vectors include recombinant prokaryotes which can infect cells and express recombinant genes. In addition to recombinant vectors, other delivery components are also contemplated such as encapsulation in liposomes, transferrin-mediated transfection and other receptor-mediated means. The invention is intended to include such other forms of expression vectors and other suitable delivery means which serve equivalent functions and which become known in the art subsequently hereto. In a preferred embodiment of the present invention, DNA is delivered to competent host cells by means of an adenovirus. One skilled in the art would readily understand this technique of delivering DNA to a host cell by such means. Although the invention preferably includes adenovirus, the invention is intended to include any virus which serves equivalent functions. In another preferred embodiment of the present invention, RNA is delivered to competent host cells by means of a retrovirus. One skilled in the art would readily understand this technique of delivering RNA to a host cell by such means. Any retrovirus which serves to express the protein encoded by the RNA is intended to be included in the present invention.

Some embodiments of the invention relate to proteins and methods of using the same. For example, in some methods of immunizing, immunogenic proteins and CD80ΔC mutant proteins are administered to individuals. Likewise, in some methods of

treating autoimmune diseases and preventing graft/transplant rejections by immunosuppression, CD80 C region proteins are administered to individuals. As used herein, the term "proteins of the invention" is intended to mean immunogenic proteins, CD80ΔC mutant proteins and CD80 C region proteins which each can be produced by similar means and which can be formulated and administered in a similar manner for use in methods of the invention.

Vectors including recombinant expression vectors that comprises a nucleotide sequence that encodes proteins of the invention can be produced routinely. As used herein, the term "recombinant expression vector" is meant to refer to a plasmid, phage, viral particle or other vector which, when introduced into an appropriate host, contains the necessary genetic elements to direct expression of a coding sequence. One having ordinary skill in the art can isolate or synthesize a nucleic acid molecule that encodes a protein of the invention and insert it into an expression vector using standard techniques and readily available starting materials. The coding sequence is operably linked to the necessary regulatory sequences. Expression vectors are well known and readily available. Examples of expression vectors include plasmids, phages, viral vectors and other nucleic acid molecules or nucleic acid molecule containing vehicles useful to transform host cells and facilitate expression of coding sequences. Some embodiments of the invention relate to recombinant expression vectors comprises a nucleotide sequence that encodes a CD80ΔC mutant protein or a CD80 C region protein. The recombinant expression vectors of the invention are useful for transforming hosts. The present invention relates to a recombinant expression vectors that comprises a nucleotide sequence that encodes a CD80ΔC mutant protein, a chimeric protein which comprises a CD80ΔC mutant protein, or a CD80 C region protein.

The present invention relates to a host cell that comprises the recombinant expression vector that includes a nucleotide sequence that encodes a CD80ΔC mutant protein, a chimeric protein which comprises a CD80ΔC mutant protein or a CD80 C region protein. Host cells for use in well known recombinant expression systems for production of proteins are well known and readily available. Examples of host cells include bacteria cells such as *E. coli*, yeast cells such as *S. cerevisiae*, insect cells such as *S. frugiperda*, non-human mammalian tissue culture cells chinese hamster ovary (CHO) cells and human tissue culture cells such as HeLa cells.

In some embodiments, for example, one having ordinary skill in the art can, using well known techniques, insert DNA molecules into a commercially available expression vector for use in well known expression systems. For example, the commercially available plasmid pSE420 (Invitrogen, San Diego, CA) may be used for production of a CD80ΔC mutant protein in *E. coli*. The commercially available plasmid pYES2 (Invitrogen, San Diego, CA) may, for example, be used for production in *S. cerevisiae* strains of yeast. The commercially available MAXBAC™ complete baculovirus expression system (Invitrogen, San Diego, CA) may, for example, be used for production in insect cells. The commercially available plasmid pcDNA I or pcDNA3 (Invitrogen, San Diego, CA) may, for example, be used for production in mammalian cells such as Chinese Hamster Ovary cells. One having ordinary skill in the art can use these commercial expression vectors and systems or others to produce proteins of the invention using routine techniques and readily available starting materials. (*See e.g.*, Sambrook et al., *Molecular Cloning a Laboratory Manual*, Second Ed. Cold Spring Harbor Press (1989) which is incorporated herein by reference.) Thus, the desired proteins can be prepared in both prokaryotic and eukaryotic systems, resulting in a spectrum of processed forms of the protein.

One having ordinary skill in the art may use other commercially available expression vectors and systems or produce vectors using well known methods and readily available starting materials. Expression systems containing the requisite control sequences, such as promoters and polyadenylation signals, and preferably enhancers, are readily available and known in the art for a variety of hosts. *See e.g.*, Sambrook et al., *Molecular Cloning a Laboratory Manual*, Second Ed. Cold Spring Harbor Press (1989).

The expression vector including the DNA that encodes a protein of the invention is used to transform the compatible host which is then cultured and maintained under conditions wherein expression of the foreign DNA takes place. The protein of the invention thus produced is recovered from the culture, either by lysing the cells or from the culture medium as appropriate and known to those in the art. One having ordinary skill in the art can, using well known techniques, isolate the protein of the invention that is produced using such expression systems. The methods of purifying proteins of the invention from natural sources using antibodies which specifically bind to such protein are routine as is the methods of generating such antibodies (See: Harlow, E. and Lane, E., *Antibodies: A*

Laboratory Manual, **1988**, Cold Spring Harbor Laboratory Press which is incorporated herein by reference.). Such antibodies may be used to purifying proteins produced by recombinant DNA methodology or natural sources.

Examples of genetic constructs include coding sequences which encode a protein of the invention and which are operably linked to a promoter that is functional in the cell line into which the constructs are transfected. Examples of constitutive promoters include promoters from cytomegalovirus or SV40. Examples of inducible promoters include mouse mammary leukemia virus or metallothionein promoters. Those having ordinary skill in the art can readily produce genetic constructs useful for transfecting with cells with DNA that encodes proteins of the invention from readily available starting materials. Such gene constructs are useful for the production of proteins of the invention.

In addition to producing proteins of the invention by recombinant techniques, automated peptide synthesizers may also be employed to produce proteins of the invention. Such techniques are well known to those having ordinary skill in the art and are useful if derivatives which have substitutions not provided for in DNA-encoded protein production.

The proteins of the invention may be prepared by any of the following known techniques. Conveniently, the proteins of the invention may be prepared using the solid-phase synthetic technique initially described by Merrifield, in *J. Am. Chem. Soc.*, 15:2149-2154 (1963) which is incorporated herein by reference. Other protein synthesis techniques may be found, for example, in M. Bodanszky *et al.*, (1976) *Peptide Synthesis*, John Wiley & Sons, 2d Ed. which is incorporated herein by reference; Kent and Clark-Lewis in *Synthetic Peptides in Biology and Medicine*, p. 295-358, eds. Alitalo, K., *et al.* Science Publishers, (Amsterdam, 1985) which is incorporated herein by reference; as well as other reference works known to those skilled in the art. A summary of synthesis techniques may be found in J. Stuart and J.D. Young, *Solid Phase Peptide Synthelia*, Pierce Chemical Company, Rockford, IL (1984) which is incorporated herein by reference. Synthesis by solution methods may also be used, as described in *The Proteins*, Vol. II, 3d Ed., p. 105-237, Neurath, H. et al., Eds., Academic Press, New York, NY (1976) which is incorporated herein by reference. Appropriate protective groups for use in such syntheses will be found in the above texts, as well as in J.F.W. McOmie, *Protective Groups in Organic Chemistry*, Plenum Press, New York, NY (1973) which is incorporated herein by reference.

In general, these synthetic methods involve the sequential addition of one or more amino acid residues or suitable protected amino acid residues to a growing peptide chain. Normally, either the amino or carboxyl group of the first amino acid residue is protected by a suitable, selectively-removable protecting group. A different, selectively removable protecting group is utilized for amino acids containing a reactive side group, such as lysine.

Using a solid phase synthesis as an example, the protected or derivatized amino acid is attached to an inert solid support through its unprotected carboxyl or amino group. The protecting group of the amino or carboxyl group is then selectively removed and the next amino acid in the sequence having the complementary (amino or carboxyl) group suitably protected is admixed and reacted with the residue already attached to the solid support. The protecting group of the amino or carboxyl group is then removed from this newly added amino acid residue, and the next amino acid (suitably protected) is then added, and so forth. After all the desired amino acids have been linked in the proper sequence, any remaining terminal and side group protecting groups (and solid support) are removed sequentially or concurrently, to provide the final peptide. The peptide of the invention are preferably devoid of benzylated or methylbenzylated amino acids. Such protecting group moieties may be used in the course of synthesis, but they are removed before the peptides are used. Additional reactions may be necessary, as described elsewhere, to form intramolecular linkages to restrain conformation.

In some embodiments, proteins may be produced in transgenic animals. The present invention relates to a transgenic non-human mammal that comprises the recombinant expression vector that comprises a nucleic acid sequence that encodes a CD80ΔC mutant protein or CD80 C region protein. Transgenic non-human mammals useful to produce recombinant proteins are well known as are the expression vectors necessary and the techniques for generating transgenic animals. Generally, the transgenic animal comprises a recombinant expression vector in which the nucleotide sequence that encodes the CD80ΔC mutant protein or CD80 C region protein is operably linked to a mammary cell specific promoter whereby the coding sequence is only expressed in mammary cells and the recombinant protein so expressed is recovered from the animal's milk. One having ordinary skill in the art using standard techniques, such as those taught in U.S. Patent No. 4,873,191

issued October 10, 1989 to Wagner and U.S. Patent No. 4,736,866 issued April 12, 1988 to Leder, both of which are incorporated herein by reference, can produce transgenic animals which produce a CD80ΔC mutant protein or CD80 C region protein. Preferred animals are goats, and rodents, particularly rats and mice.

Conservative substitutions of amino acid sequences of proteins of the invention are contemplated. As used herein, the term "conservative substitutions" is meant to refer to amino acid substitutions of CD80 residues with other residues which share similar structural and/or charge features. Those having ordinary skill in the art can readily design proteins of the invention with conservative substitutions for amino acids based upon well known conservative groups.

The pharmaceutical compositions of the present invention may be administered by any means that enables the active agent to reach the agent's site of action in the body of a mammal. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, transdermal), oral or parenteral. Because peptides are subject to being digested when administered orally, oral formulations are formulated to enterically coat the active agent or otherwise protect it from degradation in the stomach (such as prenuetralization). Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, pulmonary administration, e.g., by inhalation or insufflation, or intrathecal or intraventricular administration. In preferred embodiments, parenteral administration, i.e., intravenous, subcutaneous, transdermal, intramuscular, is ordinarily used to optimize absorption. Intravenous administration may be accomplished with the aid of an infusion pump. The pharmaceutical compositions of the present invention may be formulated as an emulsion.

One skilled in the art would readily comprehend the multitude of pharmaceutically acceptable media that may be used in the present invention. Suitable pharmaceutical carriers are described in *Remington's Pharmaceutical Sciences*, A. Osol, a standard reference text in this field, which is incorporated herein by reference. Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers,

aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Compositions for parenteral, intravenous, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives and are preferably sterile and pyrogen free. Pharmaceutical compositions which are suitable for intravenous administration according to the invention are sterile and pyrogen free. For parenteral administration, the peptides of the invention can be, for example, formulated as a solution, suspension, emulsion or lyophilized powder in association with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Liposomes and nonaqueous vehicles such as fixed oils may also be used. The vehicle or lyophilized powder may contain additives that maintain isotonicity (e.g., sodium chloride, mannitol) and chemical stability (e.g., buffers and preservatives). The formulation is sterilized by commonly used techniques. For example, a parenteral composition suitable for administration by injection is prepared by dissolving 1.5% by weight of active ingredient in 0.9% sodium chloride solution

The pharmaceutical compositions according to the present invention may be administered as a single dose or in multiple doses. The pharmaceutical compositions of the present invention may be administered either as individual therapeutic agents or in combination with other therapeutic agents. The treatments of the present invention may be combined with conventional therapies, which may be administered sequentially or simultaneously.

Dosage varies depending upon known factors such as the pharmacodynamic characteristics of the particular agent, and its mode and route of administration; age, health, and weight of the recipient; nature and extent of symptoms, kind of concurrent treatment, frequency of treatment, and the effect desired. Formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Usually, the dosage of peptide can be about 1 to 3000 milligrams per 50 kilograms of body weight; preferably 10 to 1000 milligrams per 50 kilograms of body weight; more preferably 25 to

800 milligrams per 50 kilograms of body weight. Ordinarily 8 to 800 milligrams are administered to an individual per day in divided doses 1 to 6 times a day or in sustained release form is effective to obtain desired results.

Depending upon the method for which the protein or proteins are being administered, the pharmaceutical compositions of the present invention may be formulated and administered to most effectively. Modes of administration will be apparent to one skilled in the art in view of the present disclosure.

The methods of the present invention are useful in the fields of both human and veterinary medicine. Accordingly, the present invention relates to genetic immunization of mammals, birds and fish. The methods of the present invention can be particularly useful for mammalian species including human, bovine, ovine, porcine, equine, canine and feline species.

The Examples set out below include representative examples of aspects of the present invention. The Examples are not meant to limit the scope of the invention but rather serve exemplary purposes. In addition, various aspects of the invention can be summarized by the following description. However, this description is not meant to limit the scope of the invention but rather to highlight various aspects of the invention. One having ordinary skill in the art can readily appreciate additional aspects and embodiments of the invention.

EXAMPLE

In an effort to determine why CD86, but not CD80, is required for augmentation of T-cell responses, and to further study the structure function analysis of CD80 and CD86 which has revealed several critical areas involved in binding to CD28 and CTLA-4 including residues found on both the V- and C-domains of CD80, the role of different regions of CD80 and CD86 molecules in T-cell activation were examined using coimmunization of mice with DNA immunogen and DNA encoding chimeric or truncated forms of CD80 and CD86 molecules.

Methods

Preparation of constructs:

A DNA vaccine construct encoding for the HIV-1_{MN} envelope protein (pcEnv) was prepared as described in U.S. Patent No. 5,593,972 . Human CD80 and CD86 genes were cloned from B cell cDNA library (Clontech, Palo Alto, CA) and placed into pSRαneo1+, an expression vector. More specifically, both CD80 and CD86 genes were PCR amplified as described in Kim, et al. (1997) Nature Biot. 15:641-645, which is incorporated herein by reference, and ligated into pSRαneo1+ downstream of the SRα promoter to make pCD80 and pCD86 expression vectors. The chimeric and truncated variants of these two genes were generated by PCR amplification using the Expand™ High Fidelity Polymerase system (Boehringer-Mannheim, Germany). For construction of all these forms of costimulatory molecules, pCD80 or pCD86 were used as the PCR templates. The following primers have been used in these reactions:

A: CTGCTTGCTCAACTCTACGTC - SEQ ID NO:1 (forward, vector)

B: CTGAAGTTAGCTTTGACTGATAACG - SEQ ID NO:2 (reverse, CD80)

C: GCAATAGCATCACAAATTTCA - SEQ ID NO:3 (reverse, vector)

D: CAGTCAAAGCTAACTTCAGTCAACC - SEQ ID NO:4 (forward, CD86)

E: GGGAAGTCAGCAAGCACTGACAGTTC - SEQ ID NO:5 (reverse, CD86)

F: TCAGTGCTTGCTGACTTCCCTACACC - SEQ ID NO:6 (forward, CD80)

G: TCTTGCTTGGCTTTGACTGATAACGTCAC - SEQ ID NO:7 (reverse, CD80)

H: TCAGTCAAAGCCAAGCAAGAGCATTTTCC - SEQ ID NO:8 (forward, CD80)

I: TCCTCAAGCTCAAGCACTGACAGTTC - SEQ ID NO:9 (reverse, CD86)

J: TCAGTGCTTGAGCTTGAGGACCC - SEQ ID NO:10 (forward, CD86)

K: TCTGGATCCTCATCTTGGGGCA - SEQ ID NO:11 (reverse, CD80)

L: TCTGGATCCTCATTTCCATAG - SEQ ID NO:12 (reverse, CD86)

The V-domain of CD80 was amplified using A and B primers, the C-domain, transmembrane (TM) and cytoplasmic tail (T) of CD86 were amplified using C and D primers. These fragments were then purified, combined and used as templates in second step PCR reaction using forward (CTGCTTGCTCAACTCTACGTC - SEQ ID NO:1) and reverse (GCAATAGCATCACAAATTTCA - SEQ ID NO:3) primers. The PCR product was ligated into the pSRαneo1+ vector and the resultant plasmid (pV80C86T86) encodes a chimeric costimulatory molecule expressing the V-domain of CD80 and C-, TM-, and

T-regions of CD86. The chimeric crossover point is at the conserved alanine 106 in CD80 and alanine 111 in CD86 position and respects the exon boundary.

The next plasmid pV86C80T80 which encoded CD86 V-domain and CD80 C-, TM-, and T-regions was constructed by amplification of CD86 V- region, using A and E primers. The PCR fragment encoding C, TM-, and T -domains of CD80 was amplified using C and F primers. The second stage PCR and cloning was performed as mentioned above.

Truncated forms of costimulatory molecules without C-domain (pV80CΔT80, pV86CΔT86) were also prepared by two step PCR technique. In case of pV80CΔT80 the V-domain was amplified using the A and G primers, whereas in case of pV86C(T86 V-domain was amplified using A and I primers. The TM/T fragments of both C-domain truncated molecules were amplified using C/H primers in case of pV80C(T80 and C/J in case of pV86CDT86. The resultant constructs were prepared by amplification and cloning the PCR products into the pSRaneo1+ expression vector. All constructs were verified by sequence to be faithful to the original wildtype CD80 and CD86 templates. The resulting deletion mutants lacked amino acid aspartate107 through threonine 200 in CD80 and alanine 111 through isoleucine 211 in CD86. Of note both molecules were constructed to retain 6 to 7 membrane proximal amino acids of the respective C-domain.

Finally, the T-region deletion of pCD80 (pV80C80T() and pCD86 (pV86C86TΔ) were generated by a single step PCR using in both cases A as forward and K and L as reverse primers, respectively. The PCR products were cloned into the pSR(neo1+ vector. The encoded CD80 protein terminates after the first cytoplasmic tail amino acid residue, arginine. The resulting gene for CD86 molecule terminated after nucleotide 942 preserving the first lysine in the cytoplasmic tail.

All chimeric and truncated constructs as well as the wildtype molecules were cloned into the SRαneo1+ vector. Gene expression is under the control of the SRα promoter which is composed of the simian virus 40 (SV40) early promoter and the R-segment and part of the U5 sequence (R-U5') of the long terminal repeat of human T-cell leukemia virus type 1. All constructs were verified by sequencing to be faithful to the original wildtype CD80 and CD86 templates.

Expressi n of plasmids: